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I, LEANNE MYNOTT, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PP 5733 for a patent by THE NATIONAL UNIVERSITY OF SINGAPORE filed on 04 September 1998.

I further certify that the above application is now proceeding in the name of INSTITUTE OF MOLECULAR & CELL BIOLOGY pursuant to the provisions of Section 104 of the Patents Act 1990

WITNESS my hand this
Eighth day of October 1999

L. Mynott

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PROVISIONAL SPECIFICATION

for the invention entitled:

"Therapeutic Molecules and Methods"

The invention is described in the following statement:

THERAPEUTIC MOLECULES AND METHODS

FIELD OF INVENTION

The present invention relates generally to agents useful in the treatment or prophylaxis of viral mediated disease conditions. More particularly, the present invention provides therapeutic agents useful in the treatment of cervical cancer, genital warts or asymptomatic infections caused or otherwise exacerbated by a mammalian papillomavirus (MPV). The present invention is further directed to methods of treatment using said agents as well as methods of identifying same.

BACKGROUND OF THE INVENTION

Viral mediated disease conditions represent some of the most debilitating diseases affecting humans and animals and are responsible for significant mortality and morbidity. This is particularly the case for cancers associated with viral transformation of host cells. One particularly serious form of cancer is cervical cancer. Persistent infection of the transformation zone of the cervix uteri with MPVs such as human papillomavirus (HPV) is seen as a primary cause of cervical cancer. Approximately half a million women die of cervical cancer every year, while a much higher number of patients are exposed to preinvasive disease or genital warts, and one has to conclude that treatment of these virally caused neoplasias is still inadequate in spite of the long-term establishment of surgical techniques.

MPV genomes encode proteins with molecular properties required for cellular transformation in cell culture and *in situ* . Human papillomavirus-16 (HPV-16) is the most common HPV type in malignant neoplasia and is found in about 60% of all cervical carcinomas, while about twenty other HPV types account for another 30% of these malignancies. Other HPV types that infect genital mucosa or skin, like HPV-6 and HPV-11, are most often associated

with benign neoplasia, such as genital warts.

Current treatment for HPV-16 associated lesions is surgery, while limited success is achieved for HPV-6 and HPV-11 lesions with immune modulators like interferon. Prevention of infection by HPV by vaccination and challenge of established HPV infections by immune therapy are under intense investigation, but are presently not established clinical procedures.

A need exists, therefore, for further therapeutic agents useful in the treatment or prophylaxis of disease conditions caused or exacerbated by MPVs and for methods of identifying same.

SUMMARY OF INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise" and variations such as "comprises" and "comprising" will be understood to imply the inclusion of a stated integer or step or group of integers but not the exclusion of any other integer or step or group of integers.

One aspect of the present invention provides an agent useful in the treatment or prophylaxis of a disease condition caused or exacerbated by an MPV, said agent comprising a compound capable of reducing, inhibiting or otherwise decreasing the activity of a protein encoded by an MPV gene where said agent facilitates disruption of a chelated metal cation domain present in said protein.

Still yet another aspect of the invention contemplates a composition comprising a compound capable of facilitating the disruption of a chelated metal cation domain of a protein encoded for by an MPV gene, together with a pharmaceutically acceptable carrier, diluent or excipient.

Yet a further aspect of the invention relates to a method of treating a disease condition caused or exacerbated by an MPV comprising the administration of an effective amount of a

compound capable of facilitating the disruption of a chelated metal cation domain of a protein encoded for by an MPV gene to a mammal in need thereof.

Preferably, the MPV is a human papilloma virus (HPV)

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 diagrammatically depicts the E6 protein which consists of 158 amino acids, with two Cys-X2-Cys-X29-Cys-X2-Cys zinc fingers forming the most conspicuous secondary structure. Amino acid residues shown by encircled letters are conserved among HPV-6, HPV-11, HPV-16 and HPV-18. HPV-16 and HPV-18 are the most prevalent papillomaviruses in carcinomas of the cervix precursor lesions.

Figure 2 graphically depicts the increase in RU after E6 is passed over E6BP or E6AP as measured with BIACORE technology.

Figure 3 is an autoradiographic representation of ^{35}S-Cys E6 bound to E6BP in the absence or presence of compounds.

Figure 4 is a graphic representation of quantification of ^{35}S-Cys E6 bound to E6BP or EG. The % inhibition was calculated from the DMSO value (0% inhibition or maximal binding). With 0.3% v/v H_2O_2, differential inhibition was observed for the E6-E6BP complex versus E6-dimer.

Figure 5 depicts the effective concentration for C16 under the experimental IVT-assay conditions for E6BP and E6AP. ^{35}S-Cys E6 was incubated with the indicated concentrations of C16 and assayed for complex formation with E6BP or E6AP. GST reflects the background binding of IVT E6 protein on GST-beads.

Figure 6 graphically depicts viability assays of HPV-infected cell lines incubated with E6 zinc-releasing compounds. 5000 cells/well were plated on 96 well plates and after attachment overnight, treated with 100 uM compound 2 times in 2 days before incubation with the cell proliferation reagent WST1. The cell viability was determined by absorption readings that measured the activity of mitochondrial dehydrogenase. All values were normalized to the values obtained in the presence of DMSO only.

Figure 7 graphically depicts viability assays of HPV-infected cell lines incubated with E6 zinc-releasing compounds. 10,000 cells/well were plated on 24 well plates and after attachment overnight, treated 3 times in 3 days with 50 μM compound or 10 μM C16 [C16-10]. NEM was included as a non-specific cytotoxic sulfhydryl-reacting compound. At 50 μM, C16 demonstrated significant and specific inhibition of cell viability in HPV positive cell lines (SiHa, CaSki, and HeLa), with little or no effect with HPV-negative cell lines (MCF7 and HaCat) and the nontumorigenic HeLa-fibroblast hybrid cell line 444(H444). No specific inhibition of cell proliferation was observed at 10 μM C16 (data not shown).

Figure 8 photographically depicts microscopic observation of the reduced viability of HPV-infected cell lines incubated with E6 zinc releasing compounds. Cells were treated as described for Figure 7, untreated cells were SiHa (A), HaCAt (C), HeLa (E) and 444 (G). Cells incubated with 50 uM C16 were SiHa (B), HaCat (D), HeLa (F) and 444 (H). C16 specifically effects cell morphology and cell number for SiHa and HeLa cells, with little or no effect on HaCat and 444 cells.

Figure 9 illustrates the inducement of P53 in C16 sensitive HPV-positive cell lines but not in HPV-negative cell lines. Cells were treated with C16 at 100 μM for 1 day. At the time of cell harvest most C16 treated cells were still attached to

the plate. Effects on cell morphology were observed for HeLa and SiHa cells but not for MCF7 and HaCat cells. Cells were harvested and lysed and 20 μg of total cell lysate protein was transferred to a nitrocellulose membrane and incubated with p53-antibody. Specific induction of p53 is shown for HPV-positive cell lines HeLa and SiHa after treatment with C16. No change in P53-protein level is seen for the HPV-negative cells, HaCat and MCF7, treated with C16. Note, similar induction was observed for the HPV-positive CaSki cell line. Equal loading of protein was verified with an antibody against beta-Actin.

The following single and three letter abbreviations are used for amino acid residues:

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any residue	Xaa	X

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The agents of the present invention are especially useful in the treatment of disease conditions caused by an MPV such as HPVs, for example HPV-6, HPV-11, HPV-16, HPV-18.

All HPVs have circular double stranded DNA genomes with sizes close to 8kb. The genomes of different HPV types can be aligned, and there are eight genes that are homologous among all genital HPV types. These genes contain many sequence similarities, which suggest similar and conserved (although not necessarily identical) functions. The transforming properties of HPV-16 originate from three oncoproteins that are the products of the genes E5, E6, and E7. These proteins have pleotropic effects with consequences for transmembrane signalling, regulation of cell cycle, transformation of established cell lines, immortalization of primary cell lines, and chromosomal stability (1,2). The E6 oncoprotein can form a ternary complex with the cell cycle regulator p53 and E6 associated protein, E6AP, with the result of degradation of p53 by the ubiquitination pathway (3,4). In another mechanism, E6 can bind E6BP (also called ERC-55), a calcium binding protein localized in the endoplasmic reticulum, with possible consequences for intracellular signalling (5). E6 changes cellular morphology, as it interacts with paxillin and thereby disrupts the actin cytoskeleton (6). E6 has also been described to activate (7,8) or, alternatively, repress transcription (9), to stimulate telomerase (13), to immortalize primary cell cultures (14) and to interfere with the differentiation of human keratinocytes (4).

The E6 protein of HPV-16 (Fig. 1) has a size of 158 amino acids. Its most conspicuous sequence motifs are two Cys-X2-Cys-X29-Cys-X2-Cys zinc fingers (10-13). Analysis of Swiss-Prot database indicates that this sequence motif is unique for papillomavirus E6 and E7 proteins (14), and includes numerous specific amino acids residues, highly conserved among all carcinogenic HPVs as well as many animal and human papillomavirus associated with benign lesions. The homology between all papillomavirus E6 genes permits the alignment of their nucleotide sequences, forming a useful database to establish papillomavirus taxonomy (15-17). A similar zinc finger is found in the E7 protein. The extreme conservation of E6

and E7 zinc fingers among viruses with otherwise significant sequence diversity indicates that this zinc-binding motif is required for the structure and the function of HPV E6 and E7 oncoproteins, and it has been shown that mutations affecting the HPV-16 and the bovine papillomavirus type 1 (BPV-1) E6 zinc fingers interfere with cellular transformation as well as with complex formation between E6 and E6AP and E6BP.

The structure and function of the HPV-16 E6 oncoprotein depends on the integrity of the zinc fingers, in which the sulhydryl-groups of four cysteines serving as metal-chelating residues.

The precise role of E6 in the etiology of cervical cancer is difficult to assess directly, but rather has to be inferred mostly from information on E6 function in cell culture or animals systems or molecular studies in vitro. The presently available knowledge suggests functions of E6 (and E7) in situ in three different pathological scenarios. (i) In stratified epithelia, uninfected epithelial cells differentiate without further mitoses after they left the basal and became part of the suprabasal layers. After infection by HPVs, E6 and E7 proteins interfere with this normal repression of mitosis. The consequence is a dedifferentiated and expanded cell population with HPV genomes and the progression from a clinically latent infection into a benign intraepithelial neoplasia. (ii) In these benign lesions, E6 and E7 maintain a high frequency of aberrant mitoses leading to chromosomal aberrations and aneuploidies, raising the chance for generation of increasingly tumorigenic cellular variants. (iii) Continuous expression of E6 and E7 may be required for continuous proliferation of malignant tumours and metastases. Anti-E6 and anti -E7 drugs should be able to interfere with HPV lesions on all three levels of carcinogenesis. Recently, a p53 polymorphism has been associated with the development of HPV-associated cancers. A change of residue 72 in p53 from proline to arginine results in increased degradation of p53 by HPV E6 and furthermore leads to a seven fold increase in susceptibility of homozygotes versus heterozygotes for HPV-associated cancers. Such individuals would decidedly benefit from compounds that inhibit p53 degradation by E6.

Accordingly, one aspect of the present invention provides an agent useful in the treatment or prophylaxis of a disease condition caused or exacerbated by an MPV, said agent comprising a compound capable of reducing, inhibiting or otherwise decreasing the activity of a protein encoded by an MPV gene where said agent facilitates disruption of a chelated metal cation domain present in said protein.

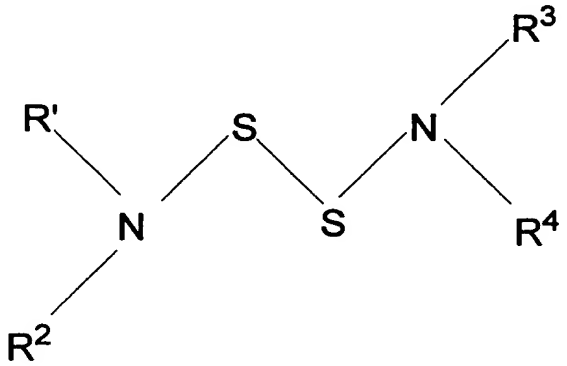
Preferably, the disease conditions to be treated are cervical cancer or precursor lesions of this malignant neoplasia, which are called cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL). The agent should also be directed to treat asymptomatic infections of the cervix in patients identified by DNA diagnosis, or asymptomatic infections that are assumed to remain after surgical treatment of cervical cancer, CIN or SIL, or asymptomatic infections presumed to exist following epidemiological reasoning. (All of these condition mentioned before are caused by HPV-16, HPV-18 and related viruses). The disease conditions to be treated also include genital warts (caused most often by HPV-6 and HPV-11), and common warts and plantar warts (caused by HPV-2 and HPV-1). All of these conditions are also caused by a large number (presently 50) of other HPV types, and The agents may also be usefully directed against these viruses. All of these lesions presumable derive from asymptomatic infections, that are most often not diagnosed. It can be assumed that The agents can also be usefully targetted against all of these asymptomatic infections.

As used herein, the term "chelated metal cation domain" refers to the structure of a protein molecule formed by chelation or association of a metal cation with two or more non-adjacent amino-acid residues. The amino acid residues may reside on a single protein molecule to form a "finger" or, alternatively, reside on different protein molecules to form, for example, a dimer. In a preferred embodiment, the metal cation is selected from manganese, iron, cobalt, nickel, copper or zinc. Most preferably, the metal is zinc. In another embodiment, the metal cation is chelated to four amino acid residues. In yet another embodiment of the invention, the metal atom is chelated to at least one cysteine residue, preferably via the sulfhydryl group.

In yet a more preferred embodiment the chelated metal cation domain is a zinc domain in which the sulfhydryl groups of four cysteine residues are chelated to the zinc cation. In still yet a more preferred embodiment, the zinc domain comprises the Cys-X2-Cys-X29-Cys-X2-Cys sequence motif.

As used herein, a protein molecule encoded for by an MPV gene refers to a peptide, polypeptide or other amino acid sequence translated from a gene in an MPV genome, or derivative thereof. Preferably, the MPV is an HPV, more preferably HPV-16 or HPV-18. In a preferred embodiment the gene is HPV-16 E6, HPV-16 E7, HPV-18 E6 or HPV-18 E7. Most preferably the gene is HPV-16 E6. Preferably, the protein is the E6 or E7 oncoprotein.

Compounds useful in the treatment of diseases and conditions caused by MPVs include compounds of the general Formula (I)



(I)

wherein

R^1 - R^4 are independently selected from hydrogen, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted arylalkyl optionally substituted acyl, optionally substituted heterocyclyl, halo alkyl, arylalkyl, carboxy, carboxy ester and carboxamido; or

R^1 and R^2 together, or R^3 and R^4 together, independently form a group of formula (a):

$$- (CH_2)_l - U_m - (CH_2)_n - \qquad (a)$$

wherein: U is selected from CH_2, O, N or S;
l and n are independently selected from 0 to 4 and m is 0 or 1 when U is CH_2 and m is 1 when U is O, N or S, such that
l+m+n is greater than or equal to 2;
and wherein any (CH_2) group may be further optionally substituted.

As used herein the term "alkyl", denotes straight chain, branched or cyclic fully saturated hydrocarbon residues. Unless the number of carbon atoms is specified the term preferably refers to C_{1-20} alkyl or cycloalkyl. Examples of straight chain and branched alkyl include methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, amyl, isoamyl, sec-amyl, 1,2-dimethylpropyl, 1,1-dimethyl-propyl, hexyl, 4-methylpentyl, 1-methylpentyl, 2-methylpentyl, 3-methylpentyl, 1,1-dimethylbutyl, 2,2-dimethylbutyl, 3,3-dimethylbutyl, 1,2-dimethylbutyl, 1,3-dimethylbutyl, 1,2,2,-trimethylpropyl, 1,1,2-trimethylpropyl, heptyl, 5-methoxyhexyl, 1-methylhexyl, 2,2-dimethylpentyl, 3,3-dimethylpentyl, 4,4-dimethylpentyl, 1,2-dimethylpentyl, 1,3-dimethylpentyl, 1,4-dimethyl-pentyl, 1,2,3,-trimethylbutyl, 1,1,2-trimethylbutyl, 1,1,3-trimethylbutyl, octyl, 6-methylheptyl, 1-methylheptyl, 1,1,3,3-tetramethylbutyl, nonyl, 1-, 2-, 3-, 4-, 5-, 6- or 7-methyl-octyl, 1-, 2-, 3-, 4- or 5-ethylheptyl, 1-, 2- or 3-propylhexyl, decyl, 1-, 2-, 3-, 4-, 5-, 6-, 7- and 8-methylnonyl, 1-, 2-, 3-, 4-, 5- or 6-ethyloctyl, 1-, 2-, 3- or 4-propylheptyl, undecyl, 1-, 2-, 3-, 4-, 5-, 6-, 7-, 8- or 9-methyldecyl, 1-, 2-, 3-, 4-, 5-, 6- or 7-ethylnonyl, 1-, 2-, 3-, 4- or 5-propylocytl, 1-, 2- or 3-butylheptyl, 1-pentylhexyl, dodecyl, 1-, 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9- or 10-methylundecyl, 1-, 2-, 3-, 4-, 5-, 6-, 7- or 8-ethyldecyl, 1-, 2-, 3-, 4-, 5- or 6-propylnonyl, 1-, 2-, 3- or 4-butyloctyl, 1-2-pentylheptyl and the like. Examples of cyclic alkyl include mono- or polycyclic alkyl groups such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclononyl, cyclodecyl and the like.

As used herein the term "alkenyl" denotes groups formed from straight chain, branched or cyclic hydrocarbon residues containing at least one carbon-carbon double bond including ethylenically mono-, di- or poly-unsaturated alkyl or cycloalkyl groups as previously defined. Unless the number of carbon atoms is specified the term preferably refers to C_{1-20} alkenyl. Examples of alkenyl include vinyl, allyl, 1-methylvinyl, butenyl, iso-butenyl, 3-methyl-2-butenyl, 1-pentenyl, cyclopentenyl, 1-methyl-cyclopentenyl, 1-hexenyl, 3-hexenyl, cyclohexenyl, 1-heptenyl, 3-heptenyl, 1-octenyl, cyclooctenyl, 1-nonenyl, 2-nonenyl, 3-nonenyl, 1-decenyl, 3-decenyl, 1,3-butadienyl, 1-4,pentadienyl, 1,3-cyclopentadienyl, 1,3-hexadienyl, 1,4-hexadienyl, 1,3-cyclohexadienyl, 1,4-cyclohexadienyl, 1,3-cycloheptadienyl, 1,3,5-cycloheptatrienyl and 1,3,5,7-cyclooctatetraenyl.

As used herein the term "alkynyl" denotes groups formed from straight chain, branched or cyclic hydrocarbon residues containing at least one carbon-carbon triple bond including ethynically mono-, di- or poly- unsaturated alkyl or cycloalkyl groups as previously defined. Unless the number of carbon atoms is specified the term preferably refers to C_{1-20} alkynyl. Examples include ethynyl, 1-propynyl, 2-propynyl, and butynyl isomers, and pentynyl isomers.

The term "heterocyclic" denotes mono- or polycarbocyclic groups wherein at least one carbon atom is replaced by a heteroatom, preferably selected from nitrogen, sulphur and oxygen. Suitable heterocyclic groups include N-containing heterocyclic groups, such as,

unsaturated 3 to 6 membered heteromonocyclic groups containing 1 to 4 nitrogen atoms, for example, pyrrolyl, pyrrolinyl, imidazolyl, imidazolinyl, pyrazolyl, pyridyl, pyrimidinyl, pyrazinyl, pyridazinyl, triazolyl or tetrazolyl;

saturated 3 to 6-membered heteromonocyclic groups containing 1 to 4 nitrogen atoms, such as, pyrrolidinyl, imidazolidinyl, piperidyl, pyrazolidinyl or piperazinyl;

condensed saturated or unsaturated heterocyclic groups containing 1 to 5 nitrogen atoms, such as, indolyl, isoindolyl, indolinyl, isoindolinyl, indolizinyl, isoindolizinyl, benzimidazolyl, quinolyl, isoquinolyl, indazolyl, benzotriazolyl, purinyl, quinazolinyl, quinoxalinyl, phenanthradinyl, phenathrolinyl, phthalazinyl, naphthyridinyl, cinnolinyl, pteridinyl,

perimidinyl or tetrazolopyridazinyl;

saturated 3 to 6-membered heteromonocyclic groups containing 1 to 3 oxygen atoms, such as tetrahydrofuranyl, tetrahydropyranyl, tetrahydrodioxinyl,

unsaturated 3 to 6-membered hetermonocyclic group containing an oxygen atom, such as, pyranyl, dioxinyl or furyl;

condensed saturated or unsaturated heterocyclic groups containing 1 to 3 oxygen atoms, such as benzofuranyl, chromenyl or xanthenyl;

unsaturated 3 to 6-membered heteromonocyclic group containing 1 to 2 sulphur atoms, such as, thienyl or dithiolyl;

unsaturated 3 to 6-membered heteromonocyclic group containing 1 to 2 oxygen atoms and 1 to 3 nitrogen atoms, such as, oxazolyl, oxazolinyl, isoxazolyl, furazanyl or oxadiazolyl;

saturated 3 to 6-membered heteromonocyclic group containing 1 to 2 oxygen atoms and 1 to 3 nitrogen atoms, such as, morpholinyl;

unsaturated condensed heterocyclic group containing 1 to 2 oxygen atoms and 1 to 3 nitrogen atoms, such as, benzoxazolyl or benzoxadiazolyl;

unsaturated 3 to 6-membered heteromonocyclic group containing 1 to 2 sulphur atoms and 1 to 3 nitrogen atoms, such as, thiazolyl, thiazolinyl or thiadiazoyl;

saturated 3 to 6-membered heteromonocyclic group containing 1 to 2 sulphur atoms and 1 to 3 nitrogen atoms, such as, thiazolidinyl, thiomorphinyl; and

unsaturated condensed heterocyclic group containing 1 to 2 sulphur atoms and 1 to 3 nitrogen

The term "acyl" denotes carbamoyl, aliphatic acyl group or acyl group containing an aromatic ring, which is referred to as aromatic acyl, or a heterocyclic ring, which is referred to as heterocyclic acyl, preferably C_{1-20} acyl. Examples of suitable acyl include carbamoyl; straight chain or branched alkanoyl such as formyl, acetyl, propanoyl, butanoyl, 2-methylpropanoyl, pentanoyl, 2,2-dimethylpropanoyl, hexanoyl, heptanoyl, octanoyl, nonanoyl, decanoyl, undecanoyl, dodecanoyl, tridecanoyl, tetradecanoyl, pentadecanoyl, hexadecanoyl, heptadecanoyl, octadecanoyl, nonadecanoyl and icosanoyl; alkoxycarbonyl such as methoxycarbonyl, ethoxycarbonyl, t-butoxycarbonyl, t-pentyloxycarbonyl and heptyloxycarbonyl; cycloalkylcarbonyl such as cyclopropylcarbonyl cyclobutylcarbonyl,

cyclopentylcarbonyl and cyclohexylcarbonyl; alkylsulfonyl such as methylsulfonyl and ethylsulfonyl; alkoxysulfonyl such as methoxysulfonyl and ethoxysulfonyl; aroyl such as benzoyl, toluoyl and naphthoyl; aralkanoyl such as phenylalkanoyl (e.g. phenylacetyl, phenylpropanoyl, phenylbutanoyl, phenylisobutylyl, phenylpentanoyl and phenylhexanoyl) and naphthylalkanoyl (e.g. naphthylacetyl, naphthylpropanoyl and naphthylbutanoyl]; aralkenoyl such as phenylalkenoyl (e.g. phenylpropenoyl, phenylbutenoyl, phenylmethacryloyl, phenylpentenoyl and phenylhexenoyl and naphthylalkenoyl (e.g. naphthylpropenoyl, naphthylbutenoyl and naphthylpentenoyl); aralkoxycarbonyl such as phenylalkoxycarbonyl (e.g. benzyloxycarbonyl); aryloxycarbonyl such as phenoxycarbonyl and napthyloxycarbonyl; aryloxyalkanoyl such as phenoxyacetyl and phenoxypropionyl; arylcarbamoyl such as phenylcarbamoyl; arylthiocarbamoyl such as phenylthiocarbamoyl; arylglyoxyloyl such as phenylglyoxyloyl and naphthylglyoxyloyl; arylsulfonyl such as phenylsulfonyl and napthylsulfonyl; heterocycliccarbonyl; heterocyclicalkanoyl such as thienylacetyl, thienylpropanoyl, thienylbutanoyl, thienylpentanoyl, thienylhexanoyl, thiazolylacetyl, thiadiazolylacetyl and tetrazolylacetyl; heterocyclicalkenoyl such as heterocyclicpropenoyl, heterocyclicbutenoyl, heterocyclicpentenoyl and heterocyclichexenoyl; and heterocyclicglyoxyloyl such as thiazolylglyoxyloyl and thienylglyoxyloyl.

The term "optionally substituted" is intended to denote that a group may or may not be further substituted or fused (so as to form a condensed polycyclic group) with one or more groups selected from alkyl, alkenyl, alkynyl, aryl, halo, haloalkyl, haloalkenyl, haloalkynyl, haloaryl, hydroxy, alkoxy, alkenyloxy, aryloxy, benzyloxy, haloalkoxy, haloalkenyloxy, haloaryloxy, nitro, nitroalkyl, nitroalkenyl, nitroalkynyl, nitroaryl, nitroheterocyclyl, amino, alkylamino, dialkylamino, alkenylamino, alkynylamino, arylamino, diarylamino, benzylamino, dibenzylamino, acyl, alkenylacyl, alkynylacyl, arylacyl, acylamino, diacylamino, acyloxy, alkylsulphonyloxy, arylsulphenyloxy, heterocyclyl, heterocycloxy, heterocycloamino, haloheterocyclyl, alkylsulphenyl, arylsulphenyl, carboxy, carboxy ester, carboxamido, carboaryloxy mercapto, alkylthio, benzylthio, acylthio, cyano, nitro , sulfate and phosphate groups.

The terms "alkoxy, "alkenoxy and "alkynoxy respectively denote alkyl, alkenyl and alkynyl groups as hereinbefore defined when linked by oxygen.

The term "halogen" denotes fluorine, chlorine, bromine or iodine.

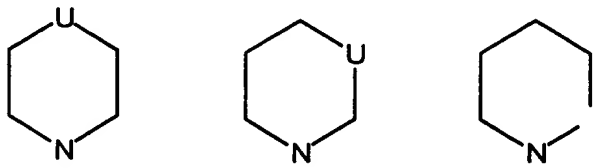
The term "aryl" denotes single, polynuclear, conjugated and fused residues of aromatic hydrocarbon ring systems. Examples of aryl include phenyl, biphenyl, terphenyl, quaterphenyl, naphthyl, tetrahydronaphthyl, anthracenyl, dihydroanthracenyl, benzanthracenyl, dibenzanthracenyl, phenanthrenyl, fluorenyl, pyrenyl, idenyl, azulenyl, chrysenyl, each of which may be further optionally substituted.

The term "haloalkyl" refers to an alkyl group, as herein before defined, substituted by one or more halogen atoms, for example, CF_3, CCl_3 or CBr_3.

The term "arylalkyl" is intended to refer to an alkyl group, as herein before defined, substituted by an aryl group, as herein before defined, for example, benzyl, ethylphenyl.

In a preferred embodiment, when R^1 and R^2 together, or R^3 and R^4 together, independently form a group of formula (a), U is CH_2 and m is 1. More preferably, the group of formula (a) is selected from one of $-(CH_2)_2-$, $-(CH_2)_3-$, $-(CH_2)_4-$,or $-(CH_2)_5-$. In yet another embodiment, the alkylenyl chain formed by $-(CH_2)_l-U_m-(CH_2)_n-$ is mono-or di- substituted at one or more $-CH_2-$ groups by an optional substituent, as herein before defined, for example; methyl, ethyl, n-propyl, iso-propyl, hydroxy, halo, methoxy, ethoxy, iso-propoxy, acetoxy, and phenyl.

In another preferred embodiment of formula (a), U is N, O, or S and m is 1. More preferably, R^1 and R^2, or R^3 and R^4, together with the nitrogen to which they are attached form a group, which may be optionally substituted as hereinbefore described, selected from:



A preferred group is the morpholino group. The compound of Formula (I) as an active ingredient may be administered in a single dose or a series of doses. While it is possible for the active ingredient to be administered alone, it is preferable to present it as a composition, preferably as a pharmaceutical composition.

Accordingly, yet another aspect of the invention contemplates a composition comprising a compound capable of facilitating the disruption of a chelated metal cation domain of a protein encoded for by an MPV gene, together with a pharmaceutically acceptable carrier, diluent or excipient.

The carrier must be pharmaceutically "acceptable" in the sense of being compatible with the other ingredients of the composition and not injurious to the subject. Compositions include those suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parental (including subcutaneous, intramuscular, intravenous and intradermal) administration.

The compositions may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, sachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g inert diluent, preservative disintegrant (e.g. sodium starch glycolate, cross-linked polyvinyl pyrrolidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

Compositions suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured base, usually sucrose and acacia or tragacanth gum; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia gum; and mouthwashes comprising the active ingredient in a suitable liquid carrier.

Compositions for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter.

Compositions suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

Compositions suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bactericides and solutes which render the composition isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The compositions may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage compositions are those containing a daily dose or unit, daily sub-dose, as herein above described, or an appropriate fraction thereof, of the active ingredient.

It should be understood that in addition to the active ingredients particularly mentioned above, the compositions of this invention may include other agents conventional in the art having regard to the type of composition in question, for example, those suitable for oral administration may include such further agents as binders, sweeteners, thickeners, flavouring agents disintegrating agents, coating agents, preservatives, lubricants and/or time delay agents. Suitable sweeteners include sucrose, lactose, glucose, aspartame or saccharine. Suitable disintegrating agents include corn starch, methylcellulose, polyvinylpyrrolidone, xanthan gum, bentonite, alginic acid or agar. Suitable flavouring agents include peppermint oil, oil of wintergreen, cherry, orange or raspberry flavouring. Suitable coating agents include polymers or copolymers of acrylic acid and/or methacrylic acid and/or their esters,

waxes, fatty alcohols, zein, shellac or gluten. Suitable preservatives include sodium benzoate, vitamin E, alpha-tocopherol, ascorbic acid, methyl paraben, propyl paraben or sodium bisulphite. Suitable lubricants include magnesium stearate, stearic acid, sodium oleate, sodium chloride or talc. Suitable time delay agents include glyceryl monostearate or glyceryl distearate.

In yet another aspect of the invention, there is provided a method of treating a disease condition caused or exacerbated by an MPV comprising the administration of an effective amount of a compound capable of facilitating the disruption of a chelated metal cation domain of a protein encoded by an MPV gene to a mammal in need thereof.

As used herein, the term "effective amount" relates to an amount of compound which, when administered according to a desired dosing regimen, provides the desired therapeutic activity. Dosing may occur at intervals of minutes, hours, days, weeks, months or years or continuously over any of these periods. Suitable dosages lie within the range of about 0.1 ng per kg of body weight to 10 g per kg of body weight per dosage. The dosage is preferably in the range of 1 μg to 10 g per kg of body weight per dosage. More preferably, the dosage is in the range of 1 mg to 10 g per kg of body weight per dosage. In a preferred embodiment, the dosage is in the range of 1 mg to 5 g per kg of body weight per dosage. In another preferred embodiment, the dosage is in the range of 1 mg to 2 g per kg of body weight per dosage. In yet another preferred embodiment, the dosage is in the range of 1 mg to 1 g per kg of body weight per dosage.

The search for anti-viral drugs is hampered when it requires assays that monitor the complete life cycle of a virus in context of the biology of the infected cell or animal. This is primarily because these *in vivo* assays are time consuming and expensive, and chemical compounds that alter the biology of the infected cells will lead to misinterpretations. In contrast, pure viral proteins expressed from cloned genes allow the development of low-cost and efficienct assays specifically designed to measure the effects on the chemistry, structure and function of these proteins. These strategies have been successfully employed recently to identify drugs against

several viral diseases, most notable against HIV-1. Similar efforts directed against papillomaviruses are in their infancy, even though these viruses affect several million patients a year worldwide.

In order to provide an initial evaluation of the efficacy of the compounds useful in the treatment of diseases or conditions caused by MPVs, the ability of these compounds to disrupt the integrity of a chelated metal cation domain, thereby releasing the metal cation, offers a useful assay therefor.

This can be achieved by contacting a protein molecule, encoded by an MPV gene, containing a chelated metal cation domain with an effective amount of said compound for a time and under conditions sufficient to facilitate disruption of the chelated metal cation domain and directly or indirectly determining the amount of chelated metal cation released wherein the amount of chelated metal cation released is indicative of the disruption of the chelated metal cation domain.

Where the chelated metal is zinc, zinc release can be measured as an increase in the florescence of the zinc-selective fluorophore TSQ (N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide) (18) in the presence of the protein and the active compound.

Mutation analysis of the cysteines involved in coordinating zinc has demonstrated that zinc binding is a requirement for E6 interaction with E6AP and E6BP (5, 19-22). As chemical alteration of cysteine residues should have similar consequences to mutational alteration, the efficacy of compounds can also be evaluated in BIACORE and GST pulldown experiments.

Therefore, another method of identifying compounds useful in the treatment of a disease condition caused or exacerbated by an MPV comprising contacting a protein molecule, encoded by an MPV gene, containing a chelated metal cation domain with an effective amount of said compound for a time and under conditions sufficient to facilitate disruption of the chelated metal cation domain and directly or indirectly determining the absence or

otherwise of binding of said protein to a ligand, wherein the absence of binding is indicative of disruption of the chelated metal cation domain.

The invention will now be described with reference to the following non-limiting examples

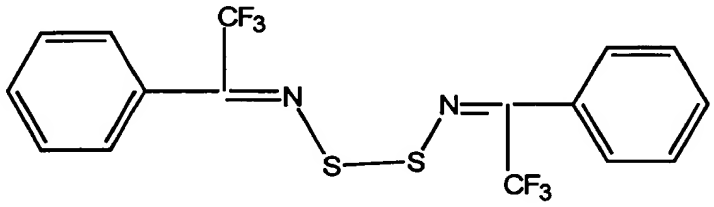
EXPERIMENTAL

In earlier studies, reduced glutathione (GSH) was required to elute recombinant glutathione sulfhydryl transferase E6 (recombinant (GST-E6)) protein during the purification process. However, the reducing activity of the GSH sulfhydryl groups protected GST-E6 protein from the chemical attack by agents, such as the disulfide based organic compounds of Formula (I) (except C16 as defined below). This problem was overcome by using GST-E6 protein in the absence of free GSH but still bound to glutathione-sepharose beads. Individual assays were done in the presence of 9 μg GST-E6 protein, corresponding to a concentration of 1 μM GST-E6 protein and 2 μM bound zinc, assuming the presence of two Zn ions per protein.

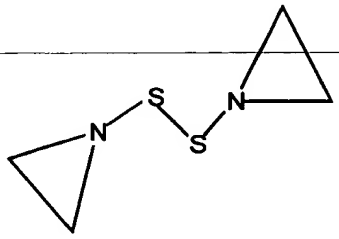
Example 1

Forty chemical compounds were evaluated as to their ability to impart structural and functional impairment of HPV-16 protein. The compounds were as follows: C1, tetraethylthiuram disulfide; C2, nitrosobenzene; C3, 4-nitrosoresorcinol-1-monomethyl ether (coniferron); C4, azodicarbonamide; C5, DL-6,8-thioctamide (alpha lipomide); C6, bis (2-nitrophenyl) disulfide; C7, 4-nitrophenyl-disulfide; C8, dibenzyl disulfide; C9, L-(-)-cystine; C10, cystamine dihydrochloride; C11, phenyl disulfide; C12, 2,2-dibenzothiazolyl disulfide; C13, 5,5′-dithiobis (2-nitrobenzoic acid) (Ellman's reagent); C14, bis (2-benzamidophenyl)disulfide; C15, 2,2′-dithiodibenzoic acid; C16, 4,4′ dithiodimorpholine; C17, 3,3′-dithiodipropionic acid dimethyl ester; C18, 2,2′-dithiodianiline; C19, DL-

homocystine; R1, 3-nitrophenyl disulfide; R2, aldrithiol-2; R4, 2-(2-nitroso-1-naphthylazo) benzoic acid; R5, 2-(carbamoylthio)-acetic acid 2-phenylhydrazide; R6, 2,2′-dithiobis-(benzothiazole); R7, 4,4′-dithiobis(N-(2-hydroxy-1-naphthylmethyl)aniline; R8, benzoyl disulfide; R9, 4-acetamidophenyl-disulfide; R10, 2-(salicylideneamino)phenyl disulfide; R11, N-{1-hydroxy-4,7-disulfo-3-[(6-aminosulfonyl phenyl)diazenyl]hydobenzo[9][7]annulen-8-yl}acetamide sodium salt; R13, azobenzene; R14,azodiisobutyrate; R15, 1,2-dithiane-4,5-diol,1,1-dioxide, cis; R16, 2,3,13,14- tetrathia-5,8-11-triaza-pentadecanebis(thioic)acid,8-(((methoxythioxomethyl)dithio) thioxomethyl)-4,12-dithioxo-,D,D-dimethylester; R17, benzoic acid,2,2′-dithiobis-,bis(((2,4-dichlorophenyl)methylene)hydrazide); R18, guanidine,N,N′′′-(dithiodi-2,1-phenylene)bis-; R19, benzoic acid, 2,2′-dithiobis-′dihydrazide; and R23 to R26 as depicted below. All compounds were dissolved in dimethysulfoxide (DMSO).



R23



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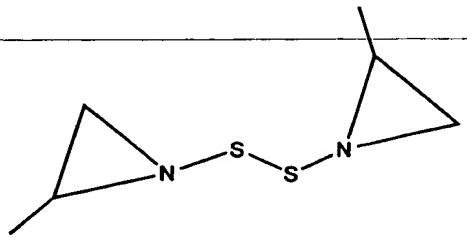

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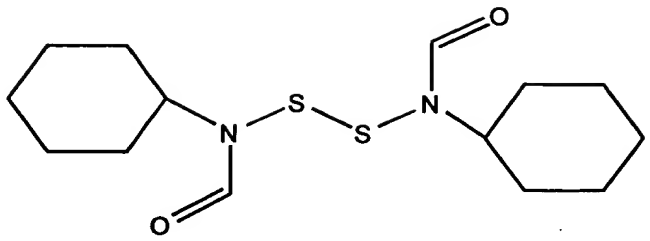

R26

E6, E6AP and E6BP-Glutathione S-transferase (GST) fusion proteins were prepared by using pGEX system (Pharmacia). The full length HPV-16 E6 gene was amplified via polymerase chain reaction and cloned in the vector pGeX4T2 as a Not1-Sal1 insert. A clone encoding the C-terminal 210 amino acids of E6BP/ERC55 in pGEX3X was a kind gift of E.J. Androphy (Chen). E6AP (amino acids 213-865), cloned in pGEX2T was a kind gift of P.M. Howley (Huibregtse). These vectors were grown in the E.coli strain AB1899, induced for fusion protein expression for 4 hrs with 0.2 mM IPTG, harvested and lysed in GST-buffer (Phosphate buffered saline (PBS), 50 mM Tris pH 8.0, 0.1% Triton) with 5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl flouride (PMSF)) and 1 mg/ml lysozyme, followed by sonication. After ultracentrifugation, supernatants of bacterial lysates were incubated at 4°C on a column of gluthathione-sepharose beads (Pharmacia). Unbound, non GST-fusion proteins were eliminated by several washes with GST-buffer. For direct use of GST-fusion proteins bound on glutathione-sepharose beads in the zinc-release assay, the glutathione-sepharose beads were resuspended in PBS, Tris pH 8.2. GST-fusion proteins for BIACORE analysis were eluted with elution-buffer (10 mM GSH, 50 mM Tris, PBS, pH 8.2)

Release of zinc from HPV-16-E6 was monitored by the change in florescence of the zinc-selective florophore TSQ (N-6-methoxy-8-quinolyl)-p-toluenesulfonamide, Molecular Probes) by modification of published procedures (25, 26). 9 μg (1 μM) recombinant GST-E6 protein, bound to glutathione-sepharose beads, were incubated with 10 μM of each compound or 0.6%

(170 mM) H_2O_2 in TSQ-assay buffer (10 mM sodium phosphate buffer pH7.0, 10% glycerol) for 2 hours at room temperature (200 μl total volume in 96-well plates). Immediately after addition of 100 μM TSQ, the increase in fluorescence was measured on a SLT Fluostar (355 nm excitation filter and 460 nm emission filter). The values were standardised with a ZnCl standard covering 0-1 μM zinc. The TSQ background values for compounds were less than the equivalent 0.1 μM ZnCl value.

Table 1 shows the values of TSQ fluorescence obtained in the presence of 1 μM ZnCl and after incubating 9 μg GST-E6 protein with an aggressive oxidizing agent, H_2O_2. A comparison of forty compounds tested for zinc release from GST-E6 shows that nine compounds produced RFU values of 50 to 75% of the H_2O_2 values, namely C4, C13, C14, C16, R2, R15, R16, R18 and R19.

TABLE 1

Values of TSQ Fluorescence

% Zinc Release			
E6-1	8.7	BEADS	12.4
E6-2	16.0	BUFFER	10.8
DMSO	9.9	1 μm ZnCl	108
C1	35.6	R1	7.6
C2	31.2	R2	**60.8**
C3	11.1	R4	12.8
C4	**61.4**	R5	20.5
C5	10.9	R6	40.8
C6	20.1	R7	14.3
C7	21.3	R8	46.7
C8	15.2	R9	40.3
C9	12.2	R10	13.8
C10	41.8	R11	15.9
C11	39.7	R13	9.8
C12	28.5	R14	33.8
C13	**61.7**	R15	**52.9**
C14	**56.1**	R16	**50.8**
C15	20.8	R17	27.4
C16	**53.5**	R18	**66.3**
C17	31	R19	**69.2**
C18	28.6	H2O2	**100**
C19	22		

The fluorescence value correlates to the amount of zinc released. The relative fluorescence units (RFU) were normalized to the amount of zinc released by H_2O_2 (100% value). E6-1 (on ice) and E6-2 (2hrs at room temperature) values represent the stability of untreated E6-GST during the incubation time. Controls include GSH-sepharose beads alone (Beads), the assay buffer alone (Buffer) and GST-E6 with DMSO. The TSQ value for 1 μM ZnCl standard is also shown. Compounds (C4, C13, C14, C16, R2, R15, R16, R18 and R19) with TSQ values higher than 50% of the H_2O_2 values were scored positive and further evaluated. All values represent the average of two independent measurements.

Example 2

Binding of GST-E6 to GST-E6BP, GST-E6AP and GST was monitored by surface plasmon resonance (SPR) on a BIACORE 2000 (Biacore AB). Purified ligand (GST, GST-E6AP and GST-E6BP) was covalently amine coupled to a CM-5 sensor chip by activation, binding and deactivation reactions suggested by Biacore AB. Typically 6000-10000 RU of GST, E6BP and E6AP were immobilized on three difference flowcells. Aliquots of purified HPV-16 GST-E6 (7 uM in 10mM GSH, 50 mM Tris/PBS buffer, pH 8.2) were incubated with either 400μM compound, or 5 mM EDTA, or 0.6% (170 mM) H_2O_2 for 2 hrs at room temperature. Then 10 μl of sample was injected at 1 μl/min over the three immobilized ligands using the sequential flow mode. The interactions between GST-E6 and ligands were monitored by the change of resonance signal in arbitrary units (RU). In between each sample, the surfaces were regenerated with a short 1 minute pulse of 50 mM NaOH that resulted in complete dissociation of all non-covalently bound analyte, leaving the immobilized GST-E6BP and GST-E6AP at approximately full activity. After 20 cycles of binding and regeneration, the amount of E6 binding capacity decreases approximately 15-25%. Typically, complex formation without compound treatment led to signals with a strength of about 1500 RU. Absence of a resonance signal, or a reduced signal was scored as an active compound, as it indicated the failure of two proteins to form heteromeric complexes. It should be noted that

10 mM reduced GSH used for the elution of GST-E6 from glutathione-sepharose beads was not removed prior to the BIACORE analysis and may inactivate some of the compounds screened. However, attempts t remove GSH resulted in GST-E6 that had weaker binding capability to GST-E6AP and GST-E6BP.

BIACORE allows real time analysis of bimolecular interactions without the need for isotopic or enzymatic labelling. BIACORE technology is based on the optical surface plasmon resonance (SPR), a technique that allows for detecting small changes in the refractive index on the surface of a thin gold film coated with a dextran matrix. Typically, one of the binding partners (termed the ligand) is covalently linked to the dextran matrix, while the other partner (termed the analyte) is introduced in a flow passing over the surface. The change in refractive index resulting from the interaction of the molecules is expressed in resonance units (RU): a SPR response of 1000 RU corresponds to a change of the surface concentration of the analyte of 1 ng protein/mm^2. GST-E6AP and GST-E6BP were used as ligands and GST-E6 was used as the analyte. Under the experimental conditions, binding between ligand and analyte typically led to SPR changes of approximately 1500-2200 RU. Controls gave the expected outcome, namely the oxidation of sulfhydryl groups by H_2O_2 of chelating of zinc ions by EDTA completely eliminated complex formation. Further controls included using the dextran matrix alone or GST as the ligand. GST-E6 did not bind significantly to the dextran matrix or to GST (background values 100 and 200 RU), respectively, excluding in the latter case the possibility of experimental errors due to interaction of the N-terminal GST residues.

Figure 2 illustrates the observations. Two of the nine compounds (C16 and R16), that had triggered zinc release from GST-E6, interfered significantly with the ability of GST-E6 to form complexes with GST-E6AP or GST-E6BP. The activity profiles of the compounds for these two cellular proteins were very similar. This was not surprising since the domain of E6BP that binds to E6 is also found in E6AP). It can be concluded that under the experimental conditions of these BIACORE assays these two compounds can trigger zinc release, resulting in alteration of the structure of GST-E6 and the corresponding failure to

establish the heterologous complexes with the two cellular proteins. The negative outcome for some of the zinc releasing compounds may lie in the presence of the relatively high concentrations of GSH (10 mM) in the BIACORE assays.

Example 3

The open reading frame of HPV-16 E6, cloned into the Hind III and PstI site of the pSP64 plasmid (Tan), was in vitro translated with ^{35}S-cysteine by using the TNT-SP6 Coupled Reticulocyte Lysate System as recommend by the manufacturer (Promega). All washing and binding reactions were performed with the E6BP-binding buffer described (Chen) but without DTT (100 mM NaCl, 100 mM Tris-HCl pH8.0, 1% NP40, 0.1% nonfat dry milk and 1 mM PMSF. 40 μl of in vitro translated E6 plus 360 μl of E6BP-binding buffer were incubated for 2hrs at room temperature with test compounds at difference concentrations from 0-1 mM (dissolved in DMSO at 1%), 5 mM EDTA, and H_2O_2 at 0.3% (85 mM). The samples were then passes over columns captaining glutathione-sepharose beads with bound GST, GST-E6, GST-E6BP or GST-E6AP proteins. The beads were heated to 95°C in 50 μl Laemmli sample buffer (BIO-RAD) with 2.5% 2-mercaptoethanol, subjected to electrophoresis on a 15% polyacrylamide gel, fixed, stained, and autoradiographed. Interference with complex formation identified reactive compounds. Desitometric quatification was performed with a BIO-RAD/GS700 imaging desitometer.

Figure 3,4 and 5 show the outcome of the binding experiments between GST-E6BP incubated with in vitro translated E6, which was treated with zinc-releasing compounds. Here, seven of the nine compounds examined in the BIACORE assays, including C16 and R16, prevented E6 protein from binding to the GST-E6BP (Fig. 3 and 4). These results further confirm the data observed in the BIACORE assays and also exclude the possibility that binding between GST-E6BP and GST-E6 protein, as measured in BIACORE assays, was due to interactions between GST-termini since GST was not present in the in vitro translated E6.

It was also observed that GST-E6 can bind to in vitro translated E6 protein (Fig. 4). This

result supports previous evidence that E6 homodimers exist in vitro, which may affect E6 function. In addition, This interaction requires E6 to be completed with zinc, as EDTA and several compounds that release zinc inhibit E6 homodimer formation. Interestingly, the activity profile of the various compounds against E6-dimer formation was quite different from that observed with E6-E6BP interaction. Only five (C4, C14, C16, R15 and R16) out of the seven compounds that inhibited E6 binding to E6BP prevented the formation of E6 homodimers. Also, while R18 and R19 had slight inhibitory activity for the E6-E6BP interaction, none was observed for E6 dimer formation. In contrast, treatment with H_2O_2 resulted in only ~45% inhibition of E6-E6BP interaction versus almost 80% inhibition of E6 dimer formation. The most dramatic difference observed was with The compound C13; while almost 80% of The E6-E6BP interaction was inhibited, none was observed for The E6 dimer interaction.

In The BIACORE results, C16 was found to have the greatest inhibitory activity of the nine compounds tested for E6 binding to both E6BP and E6AP. On this basis, it was examined whether C16 could also interfere with E6-E6BAP interaction in the GST-pulldown assay. Also, different concentrations of C16 were examined to determine the minimal concentration for inhibitory activity. As shown in Fig. 5, C16 inhibits E6 binding to both cellular proteins, with the concentration range from 10 μM (as used in the TSQ assay) to 100 μM being required for significant inhibitory activity. At 10 uM, the concentration used in the TSQ assay, little inhibitory activity is found. Only at 100 uM C16 was used in The BIACORE assays. The amount of C16 required to inhibit E6 activity in these difference assays is most likely a reflection of the amount of non-reduced C16 in the presence of endogenous or exogenous GSH.

Example 4

Zinc finger proteins are required for the maintenance of cell viability. Therefore, one concern is that the zinc-releasing compounds may have effects on normal cellular functions.

While two of the compounds tested were toxic to all the cell lines, the majority of compounds tested had little or no effect on cell growth, suggesting that there is little interference with the endogenous zinc finger proteins. At concentrations of 50-100 μM, C16 preferentially inhibited the growth of the tumorigenic HPV cell lines (SiHa, CaSki, and HeLa) as opposed to a lack of inhibition of a non-tumorigenic HPV cell line (444) and immortalized HPV-negative epithelial cell lines such as HaCat and MCF-7.

These data suggest that the specific inactivation of the E6 oncoprotein by C16 leads to a reduced viability of cell lines whose continuing growth depends on E6 functions. Decreased cell viability appears to be connected with the p53 pathway, since a dramatic increase in P53 protein levels in the C16-sensitive cell lines HeLa, SiHa, CaSki but not in the HPV-negative cell lines HaCat and MCF-7 can be demonstrated. The data indicates a link between the inactivation of HPV E6-oncoprotein, the induction of p53, and the successive occurrence of cell death. The role of p53 is not only in cell cycle control for G1-S but also in apoptosis (Polyak). Further evidence to support the model that C16 induces p53-mediated apoptosis is our recent observation that PARP cleavage, a hallmark of apoptosis, occurs in C16 treated HeLa cells.

To determine whether any of the E6 zinc-releasing compounds has a specific effect on the growth of HPV-16 containing tumour cells, we treated a variety of cell types with the nine compounds that had scored positive in the TSQ assay and measured the cell viability. As shown in Fig. 6 SiHa and CaSki cells, whose growth depends on the expression of endogenous copies of HPV-16 were examined, and HaCat cells, an immortalized human epithelial cell line that does not contain HPV. It can be seen that most of the compounds had minor unspecific effects on the viability of these cell types, with C14 and R19 having strong unspecific effects. Only one compound, C16, had strong inhibitory effects on SiHa and CaSki cells and no adverse effect on HaCat cells or MCF cells, another immortalized epithelial cell line that does not contain HPV genome (Fig.7). As a control, also examined were a variety of cell lines with C4 (Fig. 7). C4 (azodicarbonamide) causes the ejection of zinc from the p7 nucleocapsid protein of HIV and is currently in phase I/II clinical trials

against advance AIDS. No growth inhibition was observed with C4, indicating that C4 does not have specific activity against HPV-infected cells. Microscopic examination (Fig. 8a-d), revealed that C16 caused a dramatic decrease in the number of viable SiHa cells and also caused changes in the morphology of SiHa cells (similar observations were found with CaSki cells) with little change in the amount or appearance of C16-treated HaCat cells.

Examined next was the inhibitory activity of C16 on the HPV-18 containing cell lines, HeLa and 444, because the HPV-18 E6 protein shows extensive similarities to the E6 protein of the HPV-16, and may therefore be targeted by similar chemical compounds. Proliferation of HeLa cells depends on the expression of the HPV-18 E6 and E7 genes while 444 cells, which were derived from HeLa cells by fusion with fibroblasts, shown *in vivo* low expression levels of HPV-18 genes, E6 and E7 independent viability, and a lack of tumorigenicity in nude mice (23, 24). Figs. 7 and 8e-f demonstrate a strong reduction of the viability of the HeLa cells under the influence of C16, but no inhibition of 444 cultures. The most likely explanation for these results is that C16 abrogates HPV-18 E6 protein function, which adversely affects the viability of HeLa cells but leaves 444 cells uninhibited.

One potential cellular targets of E6-mediated interference of apoptosis is P53. As mentioned earlier E6 targets p53 for degradation by the ubiquitination pathway via E6AP . Therefore specific inhibition of E6-E6AP interaction with compounds might influence p53 protein levels and stability in HPV-containing cells. Therefore, we compared the p53 protein level in C16-treated cell lines (Fig. 9). The HPV-positive cell lines HeLa, SiHa and CaSki showed highly elevated levels of P53 after C16 treatment in contrast to the HPV-negative cell lines HaCat and MCF7 cells. The strongest induction was seen for HeLa cells with an increase of 80-fold in C16 treated versus untreated cells after densitometric quantification of the p53 protein levels and a 7-fold increase was observed for SiHa cells. No increase in p53 levels was found in HaCat cells which express mutant p53 and only a 2-fold increase in p53 protein levels was seen with MCF7 cells. The outcome of this experiment is in agreement with the expostulated model of an E6-mediated degradation of p53 protein in HPV-positive cell lines and elevation of p53 protein levels after inactivation of E6.

For determination of compound interference with cell viability the cervical carcinoma cell lines CaSki, SiHa (containing endogenous HPV-16 genomes) and HeLa (containing HPV-18) were analysed. The spontaneously immortalized human keratinocyte line HaCat (Boucamp) and the breast cancer derived cell line MCF-7 were used as HPV-16-negative controls. As an additional control for the comparison with HeLa cells, we used the cell line 444, a non-tumorigenic hybrid between HeLa and fibroblasts. All cell lines were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 100 U penicillin/1000 U streptomycin. Cells were allowed to attach to the surface of microwell dishes overnight. Subsequently, the medium was twice exchanged during two contiguous 24-hour periods against medium containing the zinc ejecting compounds. Viability of the cells was scored by measuring the absorption of the tetrazolium salt WST1 in an Elisa-plate reader at a wavelength of 450 nm (Cell proliferation reagent WST1 /Boehringer Mannheim).

Western Blot Analysis

10^6 cells were plated on 10 cm petri-dishes with 10 ml medium and after attachment overnight, treated with 100 μM C16 or 0.5% DMSO for one day. At the time of cell harvest most C16-treated cells were still attached to the plate. Cells were harvested and lysed in 10mM Hepes, pH 7.2, 150 mM NaCl, 0.2% NP40 and 1 mM PMSF, followed by centrifugation. 20 μg of protein was loaded onto a 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and the membrane blocked with 5% nonfat dry mile of 20 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.05% Tween-20 overnight at 4°C. The membrane was then probed with different anitbodies and the binding detected by chemiluminescence.

Role of GSH in drug-screens

GSH is, at concentrations of 1-10 mM in most cell types, the most abundant non-protein intracellular thiol, and it is involved in biochemical reactions that can inactivate pharmaceutical compounds. In the original TSQ assays, GSH was present at 5-10 mM. Under these conditions, only a few of the compounds, including C16, were capable of

releasing zinc. It was observed that physiological levels of GSH in BIACORE assays interfered with the inhibitory activity of seven out of the nine compounds that released zinc from E6 in the TSQ assay in the absence of GSH (Fig. 2). Increased concentrations of C16 were also required in the GST-pulldown assay, possibly to overcome the endogenous levels of GSH in the reticulolysate extracts. Similarly, in cell viability assays, C16 was only effective at concentrations of 50 μM, exceeding the amount used in TSQ assays five fold. To overcome the inactivating function of GSH, higher amounts of C16 were needed *in vivo* than *in vitro*. The TSQ assay is much easier when it comes to high-throughput capabilities to identify lead compounds, while *in vivo* assays and *in vitro* assays in the presence of GSH, are useful to select compounds that reach intracellular E6 in sufficiently high concentrations and in chemically unaltered form.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within the spirit and scope. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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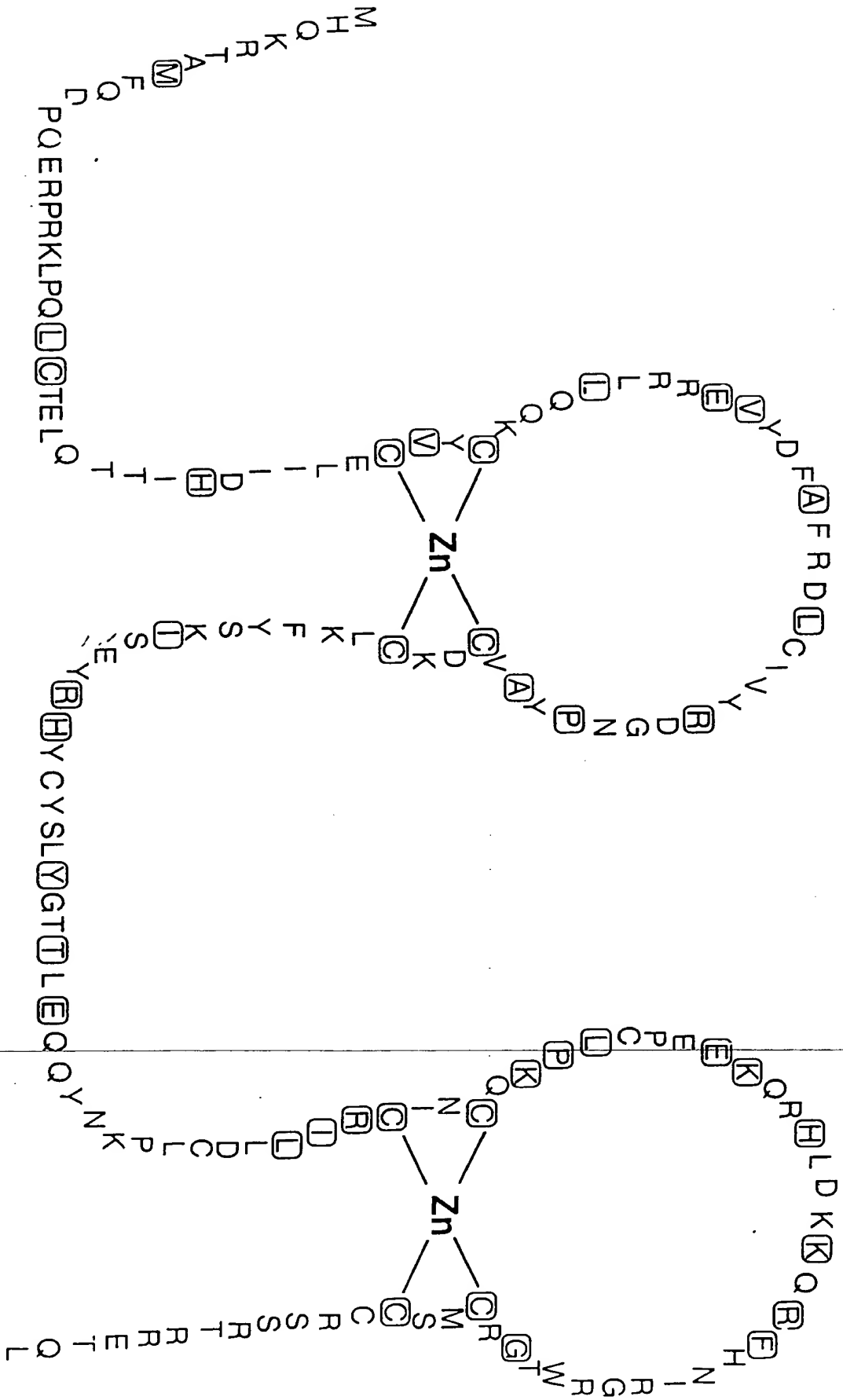
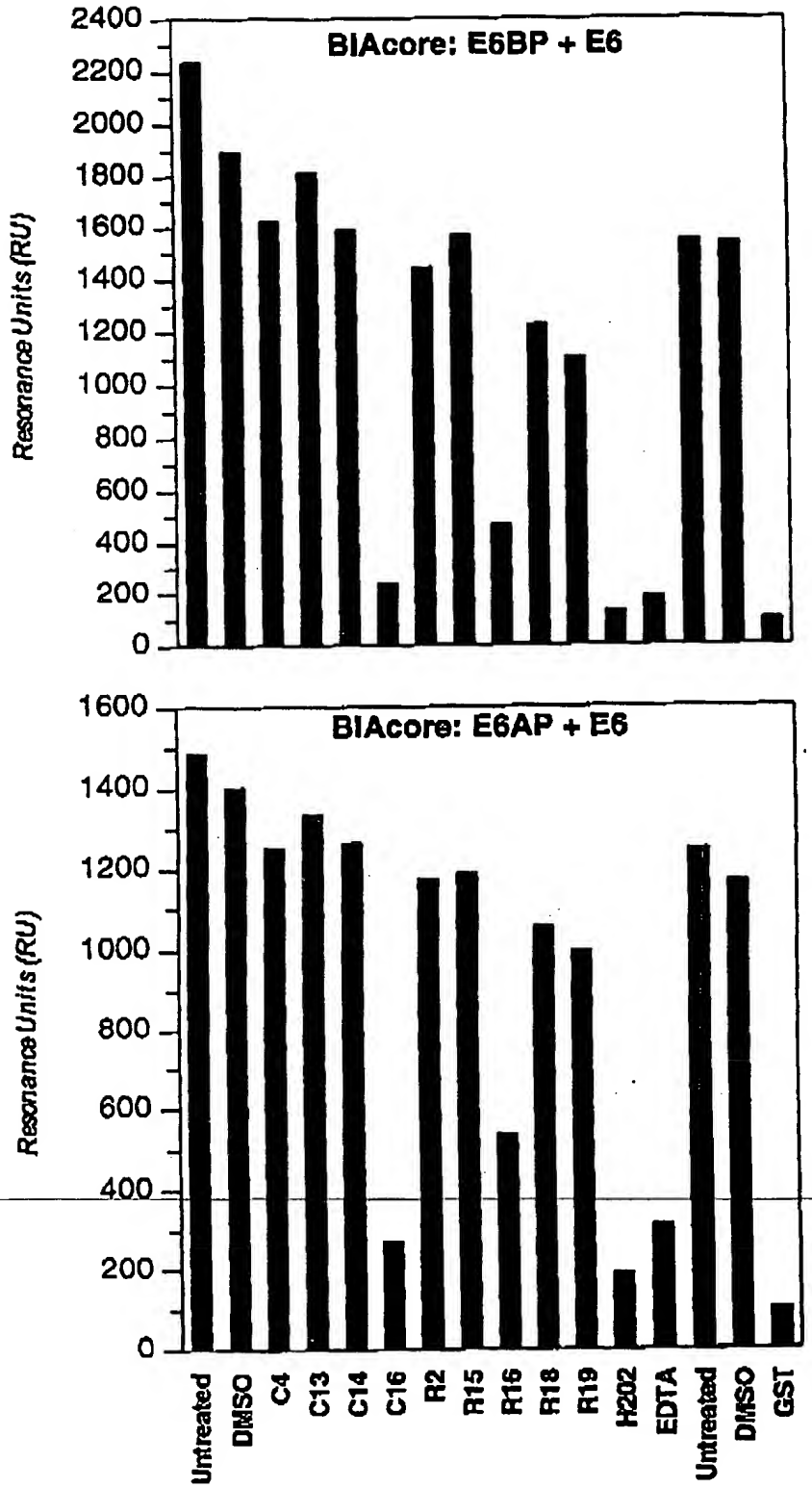


FIGURE 1.

a


BIAcore: E6BP + E6
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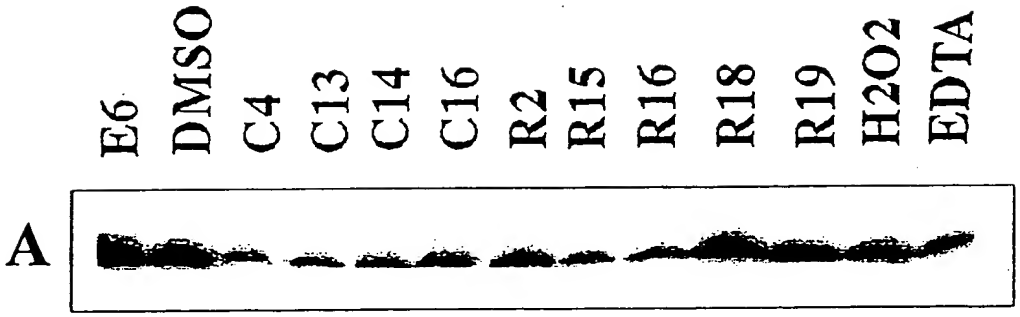
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
BIAcore: E6AP + E6
Resonance Units (RU)
1600
1400
1200
1000
800
600
400
200
0
Untreated
DMSO
C4
C13
C14
C16
R2
R15
R16
R18
R19
H202
EDTA
Untreated
DMSO
GST

Figure 2



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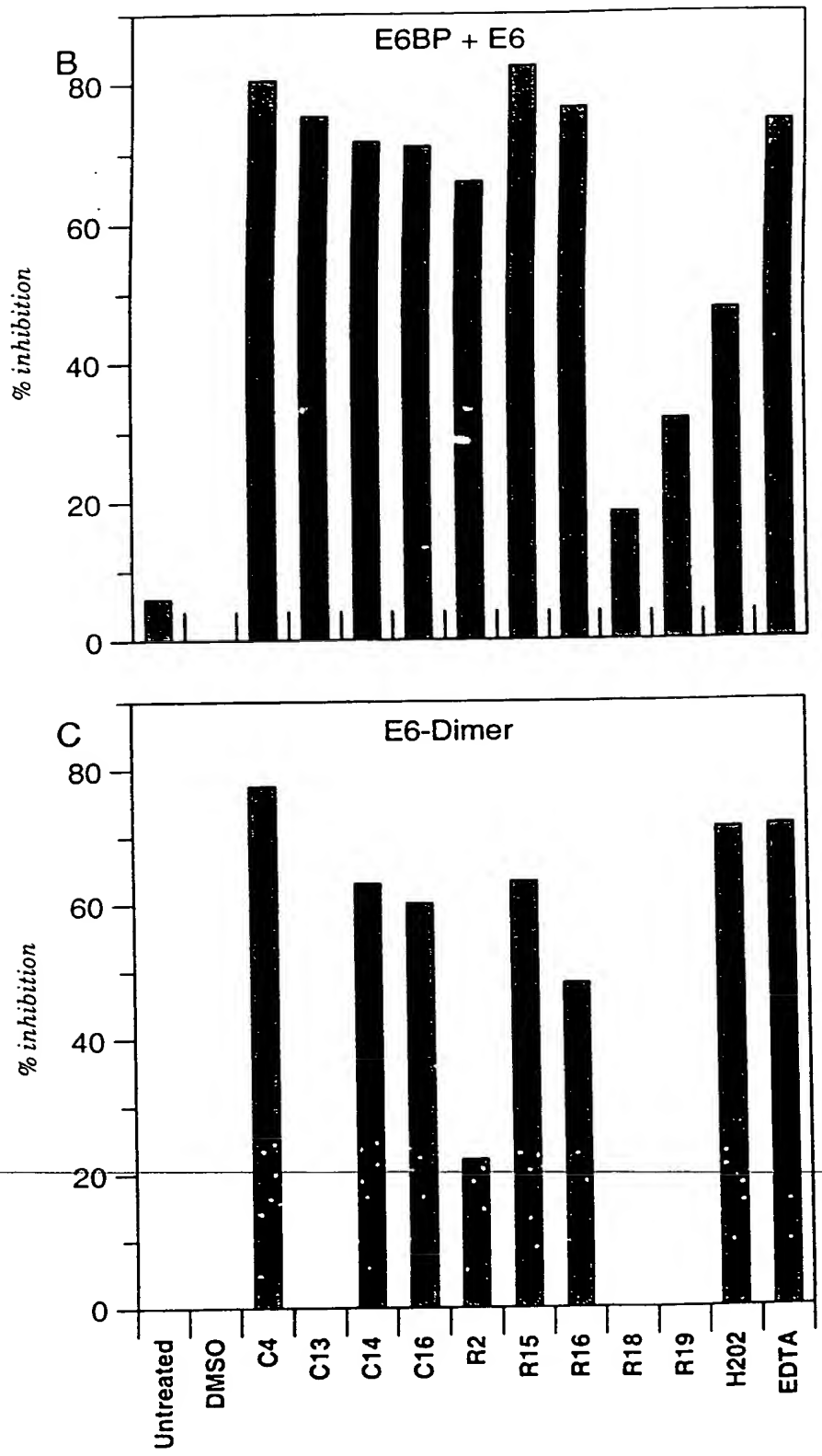
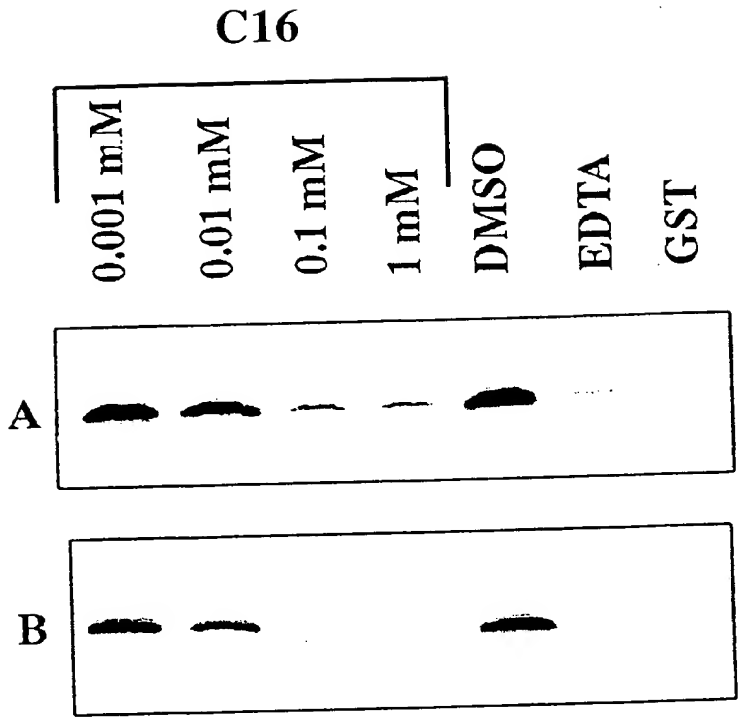

B
E6BP + E6
% inhibition
0
20
40
60
80
C
E6-Dimer
% inhibition
0
20
40
60
80
Untreated
DMSO
C4
C13
C14
C16
R2
R15
R16
R18
R19
H202
EDTA

FIGURE 4.



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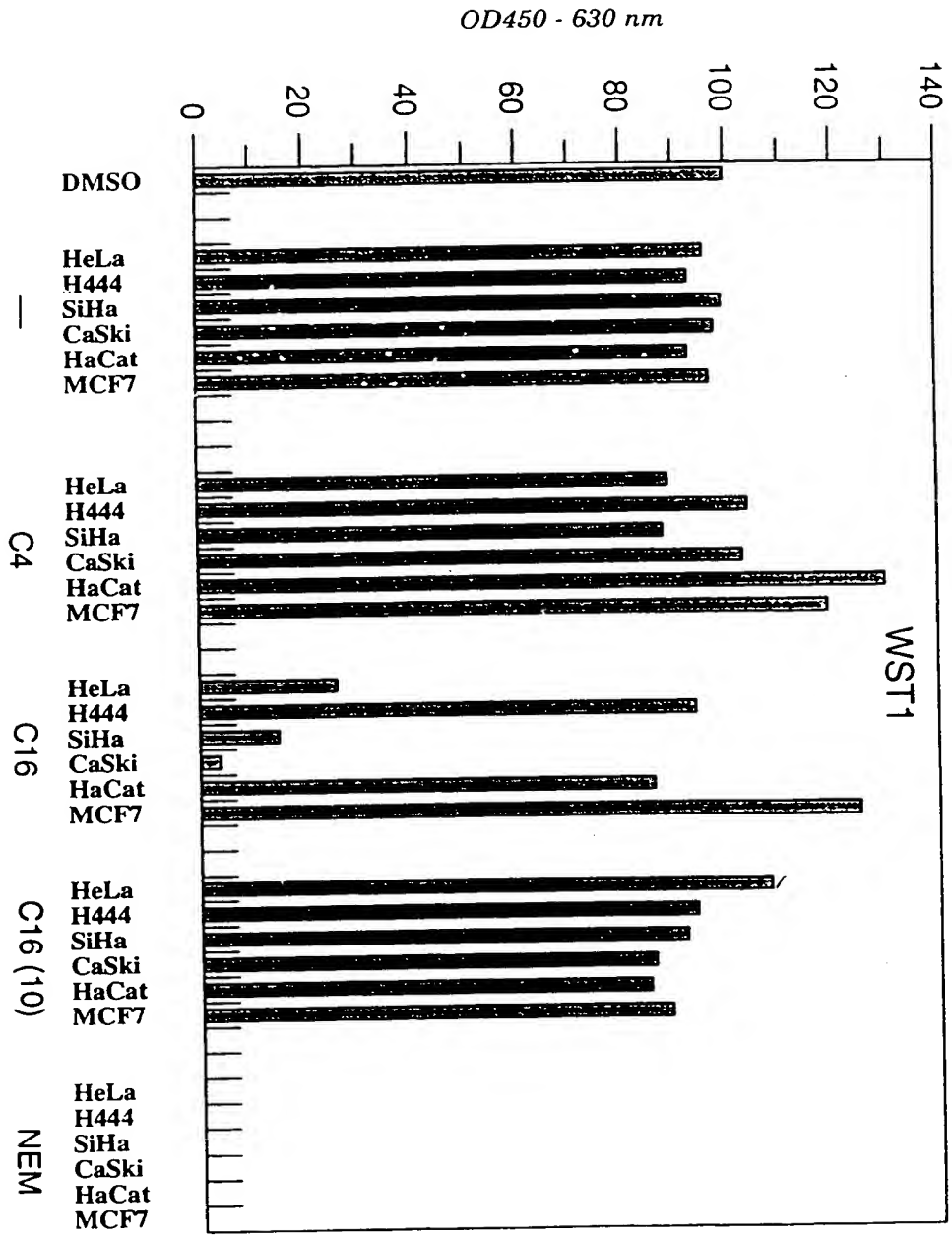
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0
20
40
60
80
100
120
140
DMSO
HeLa
H444
SiHa
CaSki
HaCat
MCF7
—
HeLa
H444
SiHa
CaSki
HaCat
MCF7
C4
HeLa
H444
SiHa
CaSki
HaCat
MCF7
C16
HeLa
H444
SiHa
CaSki
HaCat
MCF7
C16 (10)
HeLa
H444
SiHa
CaSki
HaCat
MCF7
NEM
WST1

FIGURE 6.

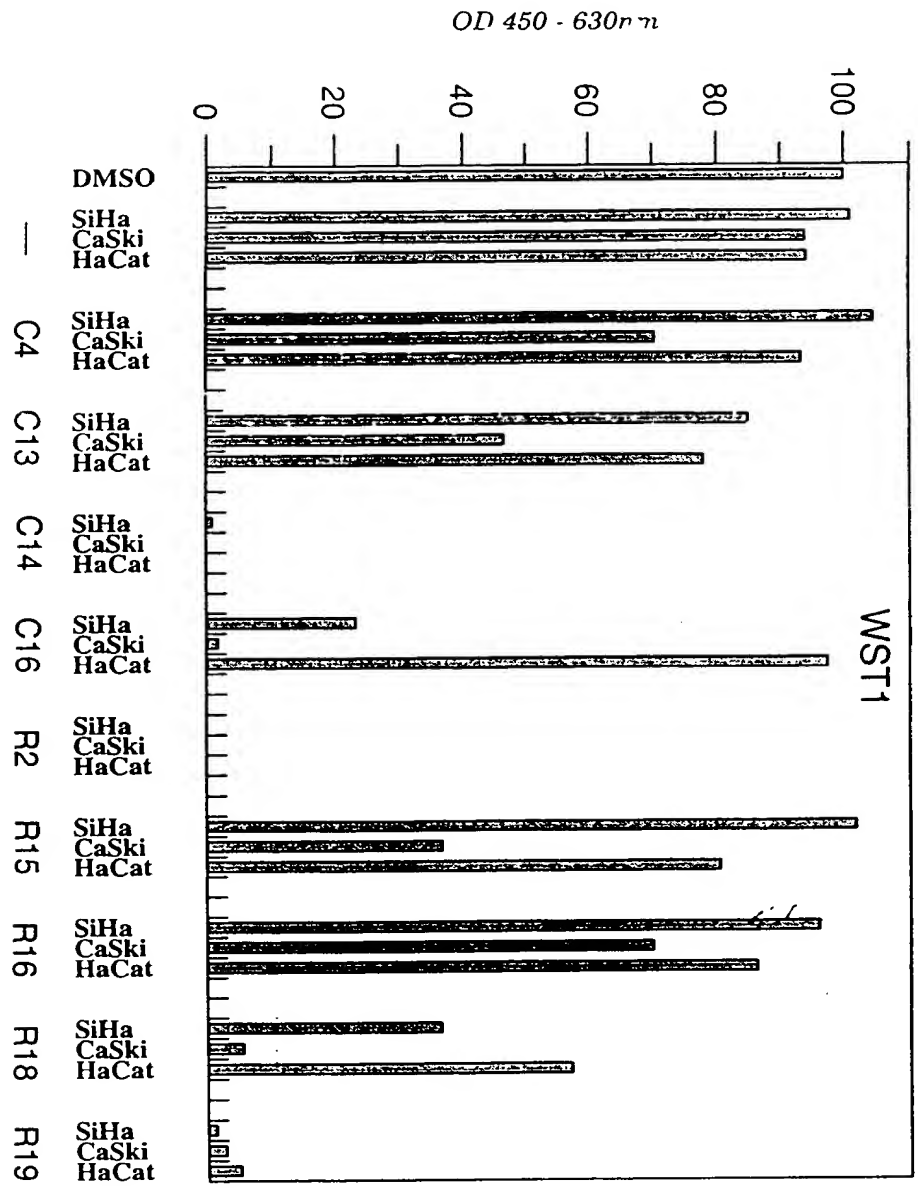
OD 450 - 630nm
0
20
40
60
80
100
WST1
DMSO
SiHa
CaSki
HaCat
—
C4
C13
C14
C16
R2
R15
R16
R18
R19

FIGURE 7.

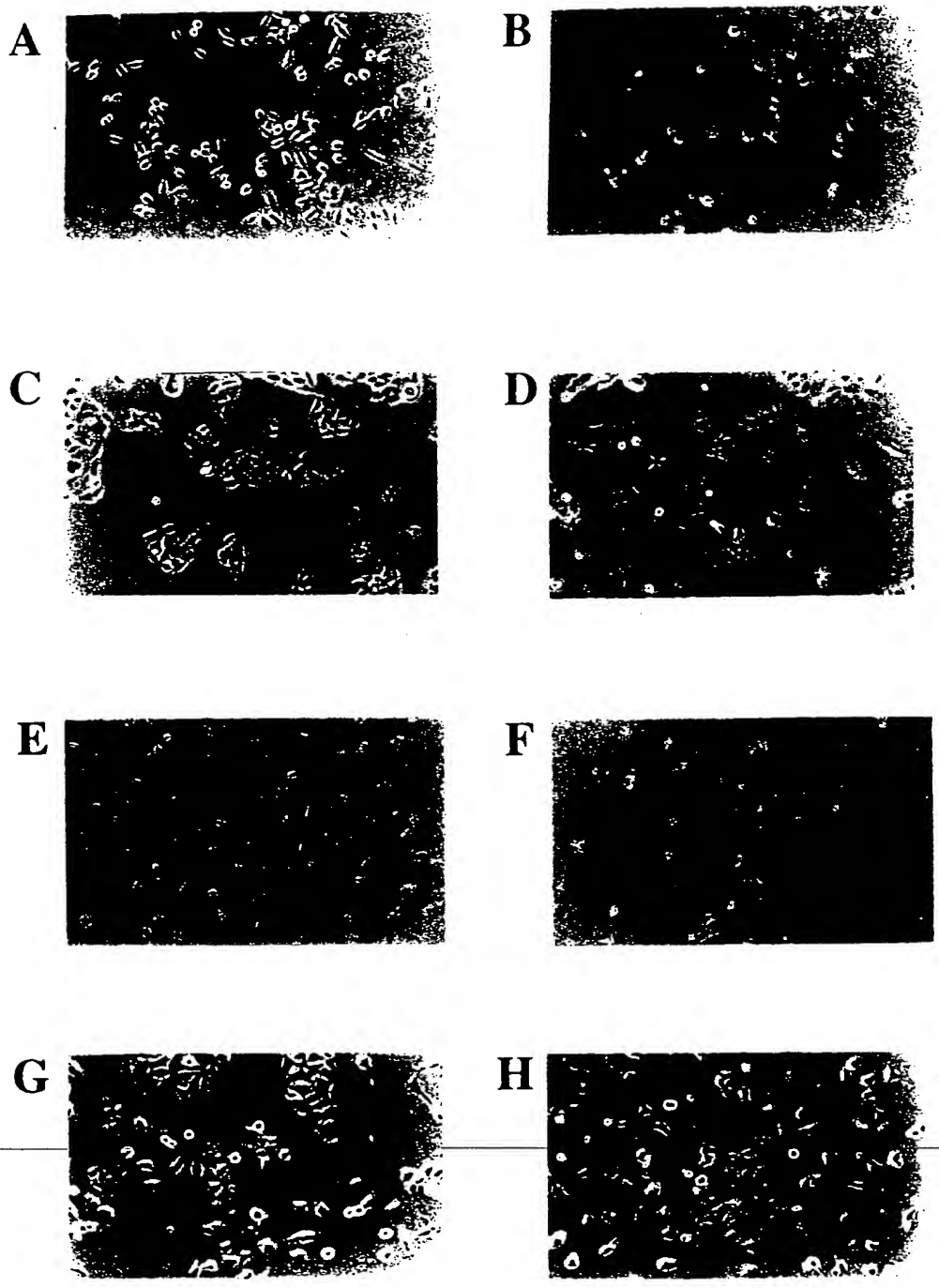

FIGURE 8.

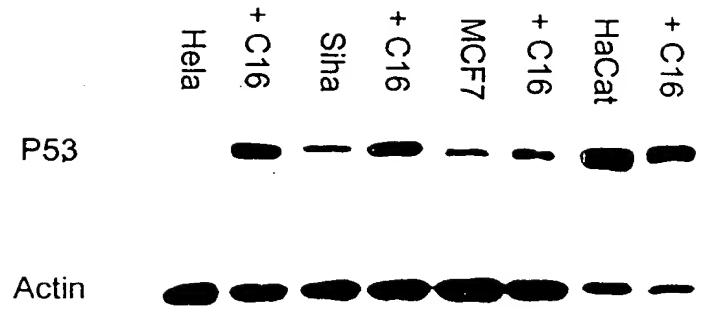

FIGURE 9.